Rec'd PCT/PTO 15 MAR 2005

PCT/AU03/01202

REC'D 30 SEP 2003
WIPO PCT

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I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2002951411 for a patent by THE UNIVERSITY OF SYDNEY as filed on 16 September 2002.

WITNESS my hand this
Twenty-third day of September 2003

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AUSTRALIA

Patents Act 1990

The University of Sydney

PROVISIONAL SPECIFICATION

Invention Title:

Genotype Screen

The invention is described in the following statement:

Field of the Invention:

The present invention relates to talent search programs for identifying individuals with elite athlete potential. More particularly, the invention relates to a method involving genotyping an individual in respect of the gene encoding the skeletal muscle protein, I-actinin 3.

Background to the Invention:

In what is an extremely competitive environment, talent search programs are increasingly being used to ensure that those with the potential to become an elite athlete are identified early in life to enable an early start in their campaign to reach the top. These talent search programs are based on actual performance data and phenotypic predictors determined by the type of training to be undertaken, as well as the likely demands from the particular sport. A weakness of both current training programs and talent search criteria is the inability to determine whether an individual has already reached his/her potential, and so is unlikely to respond optimally to further training.

Another downside of the current talent search programs, which is particularly relevant to Australia with its relatively small population base in a large geographic environment, is the opportunity for selection. That is, an individual brought up in a sporting environment is more likely to achieve success, and therefore more likely to come to the attention of coaches and talent scouts than a young individual with potential who resides in a relatively isolated location or who might otherwise have an underprivileged background. Similarly, individuals with potential to excel in lower profile sports such as rowing may be "missed" simply because such sports are less readily available in most schools, thereby diminishing the chances of early participation and notice by coaches and talent scouts. These are dilemmas facing sporting organisations such as the Australian Institute of Sport (AIS), since potential elite athletes are preferably selected and inducted into relevant training programs at a young age.

It has therefore been recognised that the identification of linkages or association of genotype or genotypic markers to certain physiological traits which can contribute or reduce performance in an elite athlete, will permit the development of DNA screens to assist in the selection of individuals with elite athlete potential. Such screens, if carried out widely, could help in overcoming some of the selection limitations of the current talent search programs discussed above. In addition, such screens may assist in recognising to whom and when a possibly very small, but critical difference in an individual's training program should be made.

The α-actinins are a family of actin-binding proteins related to dystrophin and the spectrins (Blanchard, A. et al., *Journal of Muscle Research & Cell Motility*, **10**, 280-289,

1989). In skeletal muscle, the family members α-actinin-2 and α-actinin-3 are major structural components of sarcomeric Z-lines where they function to anchor actin-containing thin filaments in a constitutive manner (Beggs, A. H. et al., *Journal of Biological Chemistry*, 267, 9281-9288, 1992). However, recent studies suggest additional roles for the α-actinins in skeletal muscle. That is, it has been found that sarcomeric α-actinins bind to other thin filament and Z-line proteins including nebulin, myotilin, CapZ and myozenin (Nave, R. et al., *FEBS Letters*, 269, 163-166, 1990, Papa, I. et al., *Journal of Muscle Research & Cell Motility*, 20, 187-197, 1999, and Salmikangas, P. et al., *Human Molecular Genetics*, 8, 1329-1336, 1999), the intermediate filament proteins, synemin and vinculin (Bellin, R. M. et al., *Journal of Biological Chemistry*, 274, 29493-29499, 1999, and McGregor, A. et al., *Biochemical Journal*, 301, 225-233, 1994), and the sarcolemmal membrane proteins, dystrophin and β1 integrin (Hance, J.E. et al., *Archives of Biochemistry & Biophysics*, 365, 216-222, 1999, and Otey, C. A. et al., *Journal of Biological Chemistry*, 268, 21193-21197, 1993) - these binding studies suggest that the α-actinins play a role in thin filament organisation and the interaction between the sarcomeric cytoskeleton and the muscle membrane. In addition, sarcomeric α-actinin binds phosphatidylinositol 4,5-bisphophate (Fukami, K. et al., *Journal of Biological Chemistry*, 269, 1518-1522, 1994), phosphatidylinositol 3 kinase (Shibasaki, F. et al., *Biochemical Journal*, 302, 551-557, 1994) and PDZ-LIM adaptor proteins (Pomies, P. et al., *Journal of Cell Biology*, 139, 157-168, 1997, and Pomies, P. et al., *Journal of Biological Chemistry*, 274, 29242-29250), suggesting a role in the regulation of myofibre differentiation and/or contraction.

In humans, the I-actinin-2 gene, *ACTN2*, is expressed in all skeletal muscle fibres, while expression of *ACTN3*, encoding I-actinin-3, is limited to a subset of type 2 (fast) fibres (North, K. N. et al., *Nature Genetics*, 21, 353-354, 1999). The present inventors have recently demonstrated that α-actinin-3 is absent in ~18% of individuals in a range of human populations and that homozygosity for a premature stop codon (577X) accounts for all cases of true α-actinin-3 deficiency identified to date. An additional polymorphism (523R) occurs in linkage disequilibrium with 577X, but does not appear to exert a deleterious effect when expressed in the heterozygous state in coupling with 577R. Further, absence of α-actinin-3 is not associated with an obvious disease phenotype, suggesting that *ACTN3* is redundant in humans (North, K. N. et al., 1999, *supra*).

Functional redundancy occurs when two genes perform overlapping functions so that inactivation of one of the genes has little or no effect on the phenotype (reviewed in Nowak, M. A. et al., *Nature*, 388, 167-171, 1997). In human skeletal muscle, α-actinin-2 expression completely overlaps α-actinin-3. Since *ACTN2* and *ACTN3* are also 80% identical and 90% similar (Beggs, A. H. et al., 1992, *supra*), and α-actinin-2 and α-actinin-3 are capable of forming heterodimers *in vitro* and *in vivo*, suggesting structural similarity and lack of significant functional differences between the two skeletal muscle α-actinin

isoforms (Chan, Y. et al., *Biochemical & Biophysical Research Communications*, 248, 134-139, 1998), it is hypothesised that α-actinin-2 is able to compensate for the absence of α-actinin-3 in type 2 (fast) fibres in humans.

Despite the apparent functional redundancy of *ACTN3* in humans, the present inventors have recently conducted genotype screens of a pool of elite Australian athletes and noted that Caucasian sprint athletes (particularly short distance runners, swimmers and cyclists) showed a very low frequency of homozygosity for the *ACTN3* premature stop codon 577X mutation (i.e. an *ACTN3* null mutation, 577XX) relative to the Australian Caucasian population at large. It is therefore considered that screening for *ACTN3* genotype, would provide considerable assistance in the selection of young Caucasian individuals with potential for elite performance in sprint-type sports and events.

Summary of the Invention:

In a first aspect, the present invention provides a method for screening individuals for elite athletic potential, the method comprising obtaining a suitable sample from an individual and determining from said sample the *ACTN3* genotype of said individual.

In a second aspect, the present invention provides a method for screening individuals for elite athletic potential, the method comprising obtaining a suitable sample from an individual and detecting in said sample, I-actinin 3 or messenger RNA encoding same.

Detailed Disclosure of the Invention:

The present inventors have identified in humans a common polymorphism in the gene encoding the skeletal muscle protein, I-actinin 3 (*ACTN3*) which is only present in type 2 (fast) fibres. There are three possible genotypes, namely 577RR (wildtype – expresses α-actinin-3), 577RX (heterozygous - α-actinin-3 present), and 577XX (homozygous null – no α-actinin-3 in skeletal muscle), and the allelic frequency varies in different ethnic groups (i.e. about 18% of Caucasians are α-actinin-3 deficient compared to ~1% African Zulus). As shown in the Example hereinafter, the present inventors have also shown that in Caucasian elite sprint athletes, the frequency of the 577XX genotype is very low, and thereby indicates that screening for *ACTN3* genotype, would provide considerable assistance in the selection of young Caucasian individuals with potential for elite performance in sprint-type sports and events.

Thus, as is mentioned above, the present invention provides a method for screening individuals for elite athlete potential, the method comprising obtaining a suitable sample from an individual and determining from said sample the *ACTN3* genotype of said individual.

Preferably, the method is employed to select, or at least assist in the selection of, young individuals with elite athlete potential.

By "elite athlete" or variants thereof, we refer to athletes which perform at the very highest levels in terms of endurance, speed and/or strength (e.g. such that they are capable of competing at State, National and/or International level in their sport).

More preferably, the method is employed to select, or at least assist in the selection of, young individuals with elite sprint athlete potential (e.g. potential as track sprinters, short distance swimmers, and track cyclists).

A suitable sample for use in the method may be any body sample including DNA, for example, a whole blood sample, a hair sample, a buccal swab or a tissue sample (e.g. muscle tissue biopsy).

Preferably, the determination of the *ACTN3* genotype is performed by amplifying (e.g. by the well known technique of polymerase chain reaction; PCR) the *ACTN3* polynucleotide sequences, or more preferably a fragment thereof which includes the 577X polymorphism (e.g. exon 16), and sequencing the amplification products or otherwise detecting the presence and/or absence of the 577X polymorphism in the amplification products. For example, the 577X polymorphism creates a *DdeI* restriction site which can be readily detected by DdeI digestion of the amplification products and thereafter determining the size of the digestion products (e.g. by separating the fragments by gel electrophoresis)).

While particularly suited to the selection of Caucasian individuals, it is considered likely that the method will also be suitable for use in selecting individuals with elite athletic potential from any other ethnic group which generally shows a high frequency (i.e. preferably at least 5%, more preferably at least 10%, and most preferably at least 15%) of the 577XX genotype. Indeed, the present inventors have also determined that the null genotype is common within the Native American population (29%), Asian population (25 %) and White Europeans (20%), PNG Highlanders (15%), African American population (13%) and the Aboriginal Australian population (10%).

Alternatively, the present invention provides a method for screening individuals for elite athletic potential, wherein the method comprises obtaining a suitable sample from an individual and detecting in said sample, I-actinin 3 or messenger RNA encoding same.

The alternative method is preferably employed to select, or at least assist in the selection of, young individuals with elite athlete potential. More preferably, the alternative method is employed to select, or at least assist in the selection of, young individuals with elite sprint athlete potential.

A suitable sample for use in the alternative method is a muscle tissue sample (e.g. muscle tissue biopsy).

Preferably, the detection of I-actinin 3 protein is by Western blot or immunocytochemistry using an I-actinin 3-specific antibody or fragment thereof (e.g. Fab fragment or a recombinant antibody fragment such as a scFv). Anti-I-actinin 3 antibodies have been previously described in North, K. N. et al., *Neuromuscular Disorders*, 6, 229-235, 1996.

Preferably, the detection of mRNA encoding I-actinin 3 is by Northern blot using a specific probe sequence, or may involve amplification (e.g. by RT-PCR) followed by sequencing of the amplification products or otherwise determining the presence of amplification products generated from mRNA encoding I-actinin 3.

Talent search programs may utilise the methods of the present invention on their own but, more preferably, in combination with similar methods for genotyping individuals in respect of other genes linked to athletic performance and/or with screening based upon performance data and phenotypic predictors (e.g height and build) and the like. Thus, the results of the methods of the present invention may be used to select, or at least assist in the selection of, young individuals with elite athlete potential and/or to provide guidance on the type of sport that a young individual may choose to specialise.

Moreover, it should be possible to devise training programs for an elite athlete which have greater chance of success, based on the knowledge of genetic factors that will predict a person's training capability. Individualised training programs would: (i) Focus on specific talents (determined from genetic makeup) by identifying the type of training which is most likely to be successful - this would help to narrow the small margin between success and failure at the elite level; (ii) Avoid unnecessary fatigue, which comes from excessive training without the expected gains (i.e. the genetic potential is not there to allow this form of training to produce the desired results in an individual) - this would reduce wasted resources and premature "burn out"; and (iii) Enhance long-term goals and self esteem – resources are wasted every time an individual with elite athlete potential has to be discarded because he/she cannot achieve success. Also, at a personal level, the effort and sacrifices already undertaken by such individuals can adversely affect their life goals and self esteem. In these situations, knowledge of the genetic makeup will help clarify why success has not been achieved, and will assist in directing the individual to more realistic life goals including more appropriate sports.

Thus, it is to be understood that the present invention extends to methods for identifying an improved training program for an athlete, involving the determination of the *ACTN3* genotype of said athlete.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element,

integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

The invention will hereinafter be described with reference to the following non-limiting example.

Example: Screening for the ACTN3 null genotype in elite athletes.

MATERIALS AND METHODS:

Human genomic DNA was isolated from blood from a pool of elite athletes (108 endurance athletes and 83 sprint athletes), 88 African Zulu individuals and 152 control Australian Caucasian individuals, by Phenol:Chloroform extraction following cell lysis with Triton-X100 and digestion with Proteinase K. Exon 16 of ACTN3 was amplified from genomic DNA. The primers for exon 16 were: forward 5'CTGTTGCCTGTGGT AAGTGGG3' and reverse 5'TGGTCACAGTATGCAGGAGGG3' corresponding to adjacent intronic sequences. The PCR reaction cycle for the primers was: 35 cycles of 94°C for 30s and then 72°C for 1 min, with a final extension of 94°C for 10 min. The R577X alleles (codons CGA and TGA respectively) can be distinguished by the presence (577X) or absence (577R) of a *Dde* I (CTNAG) restriction site in Exon 16. 577R (wild type) PCR products have 205bp and 86bp fragments; 577X PCR products have 108bp, 97bp and 86bp fragments. Digested PCR fragments were separated by 10% polyacrylamide gel electrophoresis and stained with ethidium bromide.

RESULTS:

Results of the genotyping are shown in Table 1 below.

Table 1: Genotyping of R577X in ACTN3 in Caucasians Elite Athletes.

Strength	Sport	ID	Sport Institute	Total Number	577RR (%)	577RX (%)	577XX (%)
Endurance	Rower	RT492 - RT556	AIS	64	22 (34.4%)	28 (43.8%)	14 (21.8%)
Endurance	Triathloner	RT977 - RT989	AIS	13	3 (23.1%)	8 (61.5%)	2 (15.4%)
Endurance	Cyclist	RT990 - RT998	AIS	9	4 (44.4%)	2 (22.2%	3 (33.3%)
Endurance	Track Cyclist	KN246 - KN275	AIS	22	7 (31.8%)	7 (31.8%)	8 (36.4%)
Endurance	Marathon	KN310	AIS	1	0	0	1
Endurance	**All above**		**AIS**	**108**	**36 (33.3%)**	**45 (41.7%)**	**27 (25.0%)**
Sprint	Swimmer	RT901 - RT1018	AIS	45	17 (37.8%)	25 (55.6%)	3 (6.6%)
Sprint	Track Cyclist	KN246 - KN275	AIS	8	4 (50.0%)	3 (37.5%)	1 (12.5%)
Sprint	Athletics	KN276 - KN309	AIS	30	16 (53.3%)	13 (43.3%)	1 (3.3%)
Sprint	**All above**		**AIS**	**83**	**37 (44.6%)**	**41 (49.4%)**	**5 (6.0%)**
Africa Zulu				**88**	**69 (78.4%)**	**18 (20.5%)**	**1 (1.1%)**
Australian Caucasian Control				**152**	**46 (30.0%)**	**78 (52.0%)**	**28 (18%)**

DISCUSSION:

ACTN3 genotyping was conducted in elite athletes (i.e. individuals who perform at the highest levels in terms of endurance, speed and/or strength). Compared to controls, elite sprint athletes have a low frequency of the *ACTN3* null mutation 577XX (6% versus

18% in a control Caucasian population; $p<0.05$), similar to the trend that has been observed in the Zulu population. Since, the force-generating capacity of type 2 muscle fibres at high velocity, the speed and tempo of movements, and the capacity of the individual to adapt to exercise training, all appear to be strongly genetically influenced, it is considered that *ACTN3* genotype is likely to be a factor influencing normal variation in muscle function in the general population. *ACTN3* genotyping is therefore considered to be of considerable potential in the selection, or at least to assist in the selection, of young individuals with elite athletic potential.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this sixteenth day of September 2002

The University of Sydney
By their Patent Attorneys
Blake Dawson Waldron Patent Services

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